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13. ABSTRACT *(Maximum 200 Words)*

Antizyme is a small, labile protein important in the regulation of polyamines and a likely candidate to mediate polyamine depletion as cancer therapy. Once thought of as a single protein, antizyme has been found to actually consist of at least four different forms in cells. These forms are distinct in their molecular weight, stability and responese to osmotic stress. To determine what forms originate from the first start site of AZ-1 and what post-translational modifications occur, antizyme constructs with the second start site mutated were made and transfected into rat hepatoma (HTC) and Chinese hamster ovary (CHO) cells.

Western blot analysis showed that the mutated antizyme gene, when expressed in cells, produces two proteins distinct in molecular weight. Only one band was expected, and when expressed in a cell-free system only one band was seen. The second, smaller band found in cells can only be explained by post-translational modification. These forms of antizyme behave as native antizyme with respect to activity in binding to and inhibiting ODC as well as cellular localization based on cellular fractionation experiments. These results suggests that native antizyme is post-translationally modified and this modification may play a role in the regulation of antizyme activity.

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Introduction

Antizyme is a small, labile protein important in the regulation of polyamines and a likely candidate to mediate polyamine depletion as cancer therapy. Understanding antizyme form and function and its relation to the regulation of polyamine levels in both normal and diseased cells is critical to understanding breast cancer and tumor development. The purpose of this study is to characterize the post-translational processing, cellular localization and activity of the forms of antizyme originating from the first start site of the AZ-1 gene. To this end, antizyme constructs with the second start site mutated were made and transfected into mammalian cells. Analysis of the forms of antizyme produced from the construct include: Western blots to determine forms produced, ODC-AZ assays to determine activity, and cellular fractionation to determine cellular localization.

Body

Specific Aim 1: Determine which antizyme forms originate from the first start site of the AZ-1 gene.

To determine what forms originate from the first start site of AZ-1, two antizyme constructs have been made and transfected into rat hepatoma (HTC) and Chinese hamster ovary (CHO) cells. Both constructs have the full AZ-1 gene with the following alterations: 1) a mutation (one conservative [Met34 to Ser] and one non-conservative [Met34 to Arg]) at the second start site, eliminating the possibility of translation beginning there; 2) a 6-His tag at the C-terminus to distinguish inserted from native antizyme, since there are no antizyme-negative cells available to serve as host for transfection, and; 3) a T deletion to obviate the +1 frameshift necessary for translation of native antizyme, eliminating the polyamine requirement for expression.

Because over-production of antizyme results in inhibition of cell growth, an expression system with strong regulation is required to keep this gene in the repressed state until exposed to an inducer. For this reason, these antizyme constructs were inserted into the LacSwitch II inducible mammalian expression system, which reportedly keeps inserted genes tightly repressed until exposed to IPTG, which allows rapid induction of the inserted gene. This system, however, proved to be "leaky", allowing expression of the inserted antizyme gene in the absence of inducing agent. A stable clone with moderate inducibility was obtained, but after several passages, the clone lost its inducibility. This was presumably due to the combined effects of antizyme on cell growth and the "leakiness" of the expression system.

Since loss of inducibility was attributed to the "leakiness" of the expression system, a more tightly regulated expression system, GeneSwitch was obtained from Invitrogen. This system does not have as high induction capacity as LacSwitch II, but reports much tighter repression in the uninduced state. GeneSwitch is a two-component system, which has both a 6-His tag and a V5 epitope in the expression plasmid. The antizyme constructs were inserted into the GeneSwitch expression system and HTC and CHO cells were transfected. Stable transfectants were selected by Zeocin and hygromycin resistance and clones were selected based on high inducibility.

Production of the construct antizyme protein (referred to as "mutated antizyme") was induced with Mifepristone and Western blot analysis, using anti-antizyme and anti-V5 antibodies revealed two forms in both cell types for both antizyme constructs

(conservative and nonconservative). Antibodies specific for the 6-His tag have not been useful due to the requirement for large amounts of protein required for detection. Antizyme is normally a scarce protein and even though expression of inserted constructs are much higher than seen for induced native antizyme, the levels of expression are not high enough to see with anti-6-His antibody. However, detection was possible with ant-V5 antibody when large amounts of protein were present. Also, due to the C-terminal V5 and 6-His tags, the mutated antizyme forms differ in molecular weight from native antizyme, which allows the use of antizyme specific antibodies for detection.

The full-length native antizyme results in a 29.5 kD band, whereas mutated antizyme was expected to produce a 37 kD band if no post-translational processing occurred. Indeed, when these AZ-1 constructs were expressed *in vitro* in rabbit reticulocyte lysate, only one band at 37 kD was produced. However, when expressed in tissue culture, a minor band at 37 kD and a major band at 32 kD were seen. Since there is only one start site in these constructs and the full length protein results in the larger 37 kD band, the smaller 32 kD band must be a result of proteolytic processing. This processing must be N-terminal, since the V5 epitope is C terminal, our anti-antizyme antibody recognizes the C-terminus of antizyme, and same two forms (37 and 32 kD bands) were seen after purifying the mutated antizyme via the C-terminal 6-His tag with Talon resin. Furthermore, the two mutated forms (37 kD and 32 kD) correlate with the two major native antizyme forms (29.5 kD and 24 kD) in size, being approximately 5 kD apart, and ratio, the larger form being the minor and the smaller form being the major band. These results suggest that native antizyme produced from the first start site may be processed. Also, at least part of the 24 kD band may be a result of proteolytic processing rather than a product of the second start site.

Specific Aim 2: Determine the activity of the antizyme forms produced.

The activity of the mutated AZ-1 products was determined by ODC-antizyme assays. Antizyme produced from the constructs, in both rabbit reticulocyte lysate and induced CHO cells, was purified via the 6-His tag with Talon resin and mixed with known quantities of ODC produced in HTC cells. After allowing sufficient time for antizyme to interact with ODC (15 min.) the mixture was assayed for ODC activity. The assay determines ODC activity by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine. Antizyme activity was determined by measuring the loss of ODC activity attributed to antizyme addition.

ODC-antizyme assays showed that when produced *in vitro* in rabbit reticulocyte lysate or *in vivo* in tissue culture, both antizyme constructs (conservative and nonconservative) result in protein that is active in binding and inhibiting ODC. Since the *in vitro* made protein is the larger 37 kD form and the majority of the *in vivo* made protein is the 32 kD form, and both forms contain experimentally determined sites important for ODC binding, it is likely that they are both active in binding ODC. This was reflected in the assay results.

Specific Aim 3: Determine the cellular localization of antizyme forms produced.

Cell fractionation by differential centrifugation was performed on transfected cells induced with Mifepristone and lysed by nitrogen-cavitation. Western blot analysis on each fraction, using anti-antizyme antibody was performed to determine which

fractions contained the mutated antizyme. As with native antizyme, mutated antizyme localized to every cell fraction. There was no distinction in localization with respect to form. It was expected that if the full-length protein, containing the putative mitochondrial translocation signal, were transported into the mitochondria and then the signal sequence was cleaved, the processed form would only be seen in the mitochondrial pellet. This was not seen, however approximately 20% of mutated antizyme was found to be associated with the mitochondrial pellet.

Specific Aim 4: Purify enough antizyme to sequence the N-terminus of each form produced.

Since there is no apparent difference in processing, activity or localization in conservative vs. non-conservative constructs in both HTC and CHO cells, the highest producing clone was selected for purification purposes. The highest producer of mutated antizyme was a CHO clone transfected with the non-conservatively mutated antizyme construct. Protein from this clone was purified using a metal-ion column made with Talon resin marketed by Clontech. The concentrated product was separated on SDS-PAGE and blotted to an Immobilin-PSQ membrane (Millipore). Since there were only two forms of antizyme produced and the larger form is presumably the full-length protein produced from the first Methionine, only the processed 32 kD band was selected to purify for sequencing. The 32 kD antizyme bands were cut and submitted to commercial protein micro-sequencing facilities (University of Illinois and Midwest Analytical) for evaluation of the first 7 N-terminal amino acids. Unfortunately, after repeated attempts, no antizyme sequence data was obtained, but due to the amount of protein it was concluded that the processed antizyme is N-terminally blocked.

Upon sequence analysis, it was discovered that antizyme has a 4 amino acid identity to S-Adenosyl Methionine Decarboxylase (SAMdc) and this sequence is where SAMdc is autocatalytically processed resulting in an N-terminal pyruvoyl-blocked protein. Since this 4 amino acid sequence is at a location in antizyme that could result in the processed antizyme form seen, it was concluded that antizyme might be processed and blocked in the same manner as SAMdc. Again, the same purification procedure was repeated with an immuno-precipitation step following the Talon column and the purified protein was subjected to reductive amination, which unblocks proteins with N-terminal pyruvate moieties. No antizyme sequence was obtained, which suggests that the antizyme was not unblocked by reductive amination and the blocking group on antizyme is not a pyruvate.

Further attempts will be made to unblock and sequence the processed antizyme.

Specific Aim 5: Confirm results with the use of AZ-1/GFP fusion proteins

To confirm the cellular localization results obtained by cellular fractionation, constructs of the modified AZ-1 gene, as discussed in Specific Aim 1 were made with a green fluorescent protein (GFP) tag in place of the V5 epitope. Results of laser scanning confocal microscopy (LSCM) were somewhat unexpected. Based on cellular fractionation data, it was assumed that the antizyme/GFP fusion protein would be seen throughout the cell. However, in some cells it appeared to be localized mostly to the nucleus and in other cells it appeared in a punctate pattern throughout the cytoplasm and excluded from the nucleus. Although at first, this seems to conflict with cellular

fractionation results, LSCM allows for visualization of the protein in individual cells whereas cellular fractionation results are always results of a population of cells. So, in a population of cells if a portion of that population has antizyme only in the nucleus and another portion has antizyme only excluded from the nucleus, analysis by cellular fractionation would not be able to differentiate between that and results would indicate localization to every cell part.

Since the cells visualized were clones (genetically identical), the difference between cells with nuclear localization versus nuclear-excluded localization must be some physiological difference (i.e. cell cycle event or apoptosis). To determine what this difference might be, induced cells were synchronized by mitotic shake off every hour for 24 hours and then visualized by LSCM. No apparent difference in antizyme localization was seen at any time point, which would suggest that nuclear localization versus nuclear exclusion is not a cell cycle phenomenon.

Further analysis will include induction of apoptosis as well as staining for specific organelles.

Key Research Accomplishments

- AZ-1 constructs with the second start site mutated were made, confirmed and transfected in CHO and HTC cells.
- Two forms of antizyme originating from the first start site of AZ-1constructs were identified as 37 kD and 32 kD bands, which correlate with the 29.5 kD and 24 kD forms of native antizyme.
- Antizyme activity of the protein produced from the AZ-1 constructs was determined and found to be active in binding and inhibiting ODC.
- Cellular localization of antizyme originating from the first start site of AZ-1 was determined to be comparable to that of wild-type antizyme.
- Cellular localization of AZ-1/GFP fusion protein, in individual cells, was visualized by Laser Scanning Confocal Microscopy.

Reportable Outcomes

- Satisfactory progress toward Ph.D. was made.
- CHO and HTC cells lines containing an inducible AZ-1 gene with the second start site mutated were developed.
- Participated in Biological Sciences seminar series, Northern Illinois University.
- Participated in Center for Biochemical and Biophysical Studies seminar series on Proteolysis and Protein Design, Northern Illinois University.
- Participated in 2001 Gordon Research Conference on Polyamines.
- April 2001—Presented poster entitled "Antizyme Activation in Chemotherapy and Chemoprevention", Biological Research Symposium 2001, Northern Illinois University, DeKalb, IL.
- April 2001—Phi Sigma Outstanding Graduate Research Award.
- June 2001—Presented poster entitled "Antizyme Activation in Chemotherapy and Chemoprevention", Gordon Research Conference on Polyamines, Connecticut College, New London, CT.
- February 2002—Presented poster entitled "Antizyme Activation in Chemotherapy and Chemoprevention", Office of Sponsored Projects Poster Exhibit, Northern Illinois University, DeKalb, IL.

Conclusions

The AZ-1 gene produces two forms of antizyme from the first start site, one of which is derived by proteolytic processing. These forms of antizyme behave as native antizyme with respect to activity in binding to and inhibiting ODC as well as cellular localization based on cellular fractionation experiments. This proteolytic processing may impact its function and/or localization. Additional attempts will be made to determine the cleavage site so that further characterization of the processing and its importance is possible. Surprisingly, LSCM analysis of localization of antizyme in AZ-1/GFP expressing cells showed subpopulations with antizyme found in punctate extra-nuclear or nuclear patterns. This suggests that cellular localization of antizyme may change in response to some physiological phenomenon, such as apoptosis. Antizyme activity deficiency in certain aggressive tumors lines may be correlated with abnormalities in the antizyme processing and cytolocalization.